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(54) Title: PATIENT–CONTROLLED LYMPHOCYTE METHOD FOR DETECTING NUTRIENT DEFICIENCY

(57) Abstract

Methods for detection of a nutrient deficiency in a subject are provided herein using a lymphocyte stimulation method that is patient–controlled and, therefore, is patient–specific in that the patient's own serum provides a baseline level of lymphocyte response, in addition to obtaining an optimal level of lymphocyte response, and a test level of lymphocyte response to a test nutrient. A hemotology analyzer is used to determine lymphocyte response. The invention also relates to methods for identifying nutrients that improve immune system function to assist in treatment of conditions resulting from such deficiencies, and methods for improving immune system function.

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DESCRIPTION

PATIENT-CONTROLLED LYMPHOCYTE METHOD FOR DETECTING NUTRIENT DEFICIENCY

BACKGROUND OF THE INVENTION

The immunologically active cells of the immune system are the lymphocytes, which fall into two major classes, the T lymphocytes (T cells) and the B lymphocytes (B cells). The present invention relates to the response of the T cell portion of lymphocytes to presence or absence of nutrients in an otherwise stimulatory medium for activation and initiation of growth. T cells are capable of rapid division after exposure to growth signals both in the body and in the laboratory. The growth signals and molecules (cytokines) that stimulate lymphocyte division have been well characterized. There is also an extensive literature regarding the conditions for optimal lymphocyte growth *in vitro*. It is possible to quantify cytotoxic lymphocyte function (using radioactive chromium 51 release assays), as well as lymphocyte proliferation (using radioactive thymidine incorporation into DNA).

Abnormalities of lymphocytes follow malnourishment, and deficiencies in specific nutrients have been shown to diminish lymphocyte proliferation (see e.g., Meydani, *et al.*, *Am J Clin Nutr* 1991; 53:1275-80). Most clinical nutritional analyses measure lymphocyte number or function. The measurement of lymphocyte number alone provides limited information, since there is a wide biological range of variation in human populations. For example, a patient who has a usual lymphocyte count in a well-nourished state of 3,500 cells per microliter may be malnourished with a lymphocyte count of 2,000 cells per microliter which is still in a normal range of 1,500 to 4,000 cells per microliter. At the time of clinical presentation, the usual lymphocyte count for a particular patient is generally not known, which limits the usefulness of a single measurement of lymphocyte number. Indeed, one source has concluded that total lymphocyte count is of no value as a measure of nutritional state (Rompre, *et al.*, *Can J Surg* 1985; 28:216-19).

Tests of lymphocyte function are routinely performed but are also of limited value. For instance, subcutaneous inoculation of known allergens such as tetanus toxoid, pertussis toxin, or BCG (for previous tuberculosis exposure) are performed and the skin reaction after 24 hours analyzed. This (Type IV response) is an indirect measure of helper T-cell (CD4 T cell) function. Although reduced responsivity is characteristic of malnourished patients, the prior immune status of the patient determines reactivity to any of these allergens. Therefore, a negative test may simply be indicative of non-exposure to the antigen and not of T cell functional defects. The measurement of lymphocyte number and function therefore has a low sensitivity and specificity, and, in itself, is not particularly useful as a clinical reflection of nutritional status.

These limitations have been addressed by others, for example, U.S. Patent 4,499,064 to Shive (Shive) reportedly provides an assay for assessment of nutritional status in individuals by measuring the activation and initiation of growth of lymphocytes cultured in chemically-defined serum-free medium, where various nutrients have been added or subtracted from the medium. At the end of a culture period, lymphocyte activation and initiation of growth is assayed by pulsing with tritiated thymidine and measuring thymidine incorporation into DNA utilizing a scintillation counter. The response of lymphocytes of an individual is compared with responses of lymphocytes of control individuals, or by comparing a control absent a nutrient with a test sample present the nutrient.

The lymphocyte assay of Shive can be significantly variable depending upon the time taken for the blood to reach the laboratory, the technician who performs the test on a particular day, ambient laboratory temperature, variations of nutrient levels, and carbon dioxide levels in the incubator, for example. The 24-hour pulse of tritiated thymidine is expected to provide a background level of radioactivity which would make interpretation of results difficult. In addition, the technique is complex, technically demanding, and lacks a patient-specific baseline value.

U.S. Patent 5,147,785 to Pasula reportedly provides a method for diagnosis of a malady in a subject by determining the degree of reaction between leukocytes in the subject's blood with a foreign entity having a predetermined relationship with the

malady being diagnosed. The premise of Pasula is that the reactions of a body's immune system, in particular, that the phagocytic reaction of leukocytes engulfing an entity, enlarging and then extruding its contents is basically identical in reacting to allergens as in reacting to other foreign entities recognized as harmful. Specifically, the size-distribution of the leukocytes in a control sample and a test sample of a subject's blood is compared. The test will not work, however, unless the subject has produced antibodies specific to the foreign entity or unless exogenous antibodies having specificity for the foreign entity are added. Data may be stored and used for later reference on the same subject at a later date. However, if a control sample is found to be vastly different, then further testing is cited as required to determine the cause of such a change.

These limitations have resulted in the development of methods as provided herein that are simpler, less expensive, and more accurate in detecting a nutritional deficiency.

SUMMARY OF THE INVENTION

The present invention relates generally to the fields of nutrition and health and the assessment of nutritional deficiencies. More particularly, it concerns methods for detection of a nutrient deficiency in a subject using a lymphocyte stimulation method that is patient-controlled and, therefore, is patient-specific in that the patient's own serum provides a baseline level of lymphocyte response, in addition to obtaining an optimal level of lymphocyte response, and a test level of lymphocyte response to a test nutrient. The invention also relates to methods for identifying nutrients that improve immune system function to assist in treatment of conditions resulting from nutrient deficiencies, and methods for improving immune system function.

By "patient-controlled," as used herein, is meant that the patient's lymphocytes are separately observed for proliferation in the presence of a test nutrient to provide a test count, in the presence of optimal proliferating media to provide an optimal count, and in the presence of autologous serum to provide a baseline count. In this way, the

patient sample provides a positive optimal control value and a baseline value for comparison with a test sample value and is, therefore, "patient-controlled."

By "lymphocyte method," as used herein, is meant observing proliferation of lymphocytes, i.e., a change in size and/or a change in number of lymphocytes under the above three conditions in the presence of a mitogen. "Proliferation," as used herein, means a change in size and/or a change in number of lymphocytes.

By "nutrient," as used herein, is meant a substance in the diet that furnishes nourishment to the body and includes essential dietary substances that cannot be made in the body.

By "deficiency," as used herein, is meant a level of nutrient in a subject where addition of the nutrient to the patient-controlled lymphocyte method as described herein provides proliferation of lymphocytes in an amount greater than a baseline amount. Thus, deficiency would include conditions where a subject may lack an optimal amount of nutrient, or may require an abnormal amount of a nutrient, or may have a storage inadequacy of a nutrient.

Accordingly, an embodiment of the present invention is a method for detecting a deficiency of a nutrient in a subject, the method comprising obtaining lymphocytes from the subject; obtaining serum from the subject; incubating the lymphocytes in the presence of the nutrient and a mitogen for a time period to allow at least about a doubling of lymphocyte number to provide a test sample; incubating the lymphocytes in the presence of optimal proliferating media and a mitogen for the time period to provide an optimal sample; incubating the lymphocytes in the presence of the serum and a mitogen for the time period to provide a baseline sample; counting the lymphocytes in the test sample, the optimal sample, and the baseline sample to produce a test count, an optimal count; and a baseline count; dividing the test count by the optimal count to obtain a test value; dividing the baseline count by the optimal count to obtain a baseline value; and comparing the test value with the baseline value. When the test value is greater than the baseline value, a deficiency of the nutrient is detected in the subject.

A further embodiment of the present invention is a method for detecting a deficiency of a nutrient in a subject, the method comprising obtaining lymphocytes from the subject; obtaining serum from the subject; incubating the lymphocytes in the

presence of the nutrient and a mitogen for a time period less than about a doubling time to provide a test sample; incubating the lymphocytes in the presence of optimal proliferating media and a mitogen for the time period to provide an optimal sample; incubating the lymphocytes in the presence of the serum and a mitogen for the time period to provide a baseline sample; determining size of the lymphocytes in the test sample, the optimal sample, and the baseline sample to produce a test size, an optimal size; and a baseline size; and comparing the test size with the optimal size to obtain a change value; and comparing the baseline size with the optimal size to obtain a baseline value. When the change value is greater than the baseline value, a deficiency of the nutrient is detected in the subject.

A method for improving immune system function in a subject is a further aspect of the present invention. The method comprises performing the method as herein described; and providing the deficient nutrient to the subject in an amount effective to improve immune system function.

Advantages of the methods provided herein include simplicity; less cost, increased accuracy, no radioactivity is used, a chemically-defined growth medium is not required, the calculations involved do not include complex dose response curves but simply percentages of optimal proliferation; and each patient provides internal positive and negative controls to ensure validity of the results.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The invention described herein measures the effect of nutrients on lymphocyte response by documenting an increase in size of lymphocytes after a period in culture, or by documenting the absolute number of lymphocytes after a period of cell division under conditions where a baseline value is determined that is patient-specific, thereby providing individual patient-specific and patient-relevant clinical information.

Lymphocytes from patients are separated from blood samples and then cultured for a variable time period with mitogen under at least three conditions: i) in the presence of the patient's own (autologous) serum, ii) in the presence of a test nutrient, and iii) in the presence of optimal proliferating media. At the end of the culture period,

cell number and/or changes in cell size are analyzed using a cell counter/sizer such as a hematology analyzer or a flow cytometer. Suboptimal proliferation after culture in the patient's serum demonstrates that a nutrient that is required for optimal growth is inadequately present in the patient's serum. Normalization of proliferation by addition of that nutrient demonstrates that that nutrient is present at suboptimal concentrations in the patient's serum. Addition of the nutrient to the diet of the patient is expected to result in clinical benefit. The present invention provides a direct, unambiguous, and simplified method of determining specific nutritional deficiencies in malnourished patients.

A preferred embodiment of the present method includes:

- Venous blood is collected into a vacutainer tube containing an anticlotting agent;
- A separate blood sample is collected into a standard clotting tube to provide serum which is the fluid left after blood has clotted;
- Lymphocytes are separated by centrifugation of the unclotted blood sample over a Ficoll gradient (800 g, 20 minutes), aspirated from the interface, and washed in RPMI medium (no fetal calf serum [FCS] added), (Sigma Chemical Co., St. Louis, Mo.). Alternatively, a chemical lysing reagent may be used to eliminate red cells followed by washing of remaining white cells;
- The patient's serum is collected from the clotted tube after centrifugation (400 g, 5 minutes);
- Lymphocytes are counted and medium added (RPMI, no FCS) to make up a final concentration of about 1 x 10^6 cells per ml, aliquots of 100 microliters of the cell suspension (about 100,000 cells) are then pipetted into wells of a 96-well round-bottom plate;
- Patient's serum (100 microliters, for example) is added to a plurality of wells to provide a baseline sample;
- Optimal proliferating media (100 microliters, for example), such as, normal control serum, or FCS with RPMI (FCS, Sigma Chemical Co., St. Louis, Mo.) is added to a different plurality of wells to provide an optimal sample;

- A test nutrient is added to yet a different plurality of wells at differing concentrations to provide a test sample (a 96-well plate allows for the testing of a plurality of nutrients in one set);
- A mitogen or lymphocyte stimulant is added to some of each of the above plurality of wells (some wells do not have a mitogen to serve as a negative control);
- At the end of a predetermined length of time for incubation (at about 37° C, preferably a humidified incubator, about 5% CO_2), either cell count or size of lymphocytes in each well is recorded. Cells are counted or sized using a standard cell counter/sizer, a hematology analyzer (as set forth in U.S. Patents 5,147,785, 4,788,155 or 4,614,722 (each patent is incorporated by reference herein)), or a flow cytometer , for example. Other known methods used in determining numbers of lymphocytes could also be used. Preferably, each sample is done in triplicate, and the average cell number or size is recorded.
- The data are statistically analyzed for comparisons between a test count, an optimal count, and a baseline count.

By "mitogen or lymphocyte stimulant," as used herein, means a substance that stimulates lymphocytes to undergo mitosis. The fraction of lymphocytes that respond to a mitogen is the T cell fraction. Actively dividing lymphocytes, which are called lymphoblasts, are somewhat larger than resting lymphocytes, their cytoplasm increases in volume and becomes filled with ribosomes. Their nucleus is less compact and develops nucleoli. The conversion of a resting lymphocyte into a lymphoblast is called lymphocyte transformation or blast transformation. Proliferation, as used herein, means either or both the processes of transformation and cell division. A mitogen may be a substance such as Concanavalin A (5 micrograms per ml), phytohemagglutinin (PHA) (1 to 10 micrograms per ml), irradiated allogeneic lymphocytes, or a cytokine such as interleukin-2, for example. Lymphocyte proliferation is slowed or does not occur if a nutrient is present in a suboptimal level or is deficient.

By "a predetermined length of time for incubation," as used herein, is meant a time period to allow at least about a doubling of lymphocyte number when the method is counting numbers of lymphocytes. This time period is at least about 12-24 hours, optimally, about 48 to 72 hours and may be up to 4, 5, or 6 days. This time period is

less than about a doubling time when the method is determining size of lymphocytes and, preferably, is less than about 12 hours. Size thresholds of cell counters/sizers may be set to exclude particles of greater or less than a certain size. For example, the ALCAT® test of U.S. Patents 5,147,785, 4,788,155 and 4,614,722 (each patent is incorporated by reference herein), which tests for food allergens, utilizes change in cell size after incubation with a food allergen as an indication of food sensitivity, and has been clinically validated.

A test nutrient, as defined hereinabove, is a substance in the diet that furnishes nourishment to the body and includes an essential dietary substance that cannot be made in the body. A test nutrient includes a substance such as a vitamin, a mineral, an amino acid, a fat-related molecule, a carbohydrate, a metabolite, or an anti-oxidant.

Amino acids may include any of the naturally-occurring amino acids, and, in particular, includes arginine, lysine, cysteine/cystine, glycine, leucine, isoleucine, methionine, serine, threonine, tryptophan, tyrosine, valine, glutamine or glutamic acid, for example.

Vitamins may include biotin; niacin, nicotinic acid, or niacinamide; thiamine (Vitamin B1); riboflavin (Vitamin B2); Vitamin B3; Vitamin B5 (pantothenic acid); pyridoxine, pyridoxal, or pyridoxamine (Vitamin B6); folic acid or tetrahydrofolate; cyanocobalamin (Vitamin B12); Vitamin C; Vitamin D; Vitamin E; Vitamin K; or Vitamin A; for example.

A mineral may include an electrolyte, a metallic nutrient, or a non-metallic nutrient. Electrolytes may include calcium or magnesium, for example. Metallic nutrients may include zinc, iron, copper, chromium, cobalt, manganese, nickel, germanium, vanadium, or selenium, for example. Non-metallic nutrients may include iodine, phosphorous, selenium, fluoride, boron or silicon, for example.

A fat-related nutrient may include choline, inositol, cholesterol, a triglyceride, HDL, LDL, lipid, or an essential fatty acid, for example.

A carbohydrate may include a pentose or hexose, for example.

A metabolite, as used herein, is a substance involved in metabolism, and may include pantothenic acid, carnitine, gamma-amino butyric acid, taurine, para-amino benzoic acid, a bioflavonoid, coenzyme Q10, or glutathione, for example.

An anti-oxidant, as used herein, means a substance that scavenges reactive and harmful oxygen species, such as Vitamin C, selenium, Vitamin A, or Vitamin E, for example.

An example of data that may result from the present methods is as follows: when an optimal sample count (under optimal conditions) is 200,000 lymphocytes, a baseline count (in presence of patient's serum) is 80,000 lymphocytes, and a test count (in the presence of a test nutrient, thiamine, for example) is 170,000 lymphocytes per well, the data show that thiamine has a unambiguously positive effect on lymphocyte division, thereby detecting a thiamine deficiency. If a test count in the presence of a test nutrient niacin, for example, results in a mean of 75,000 lymphocytes per well, then no positive effect on lymphocyte division is seen and no deficiency of niacin exists.

Symptoms related to a nutrient deficiency for specific nutrients are provided in Table 1.

TABLE 1

NUTRIENT	SYMPTOMS RELATED TO NUTRIENT DEFICIENCY
Vitamin B1	Beri beri, Wernig encephalopathy, alcoholism
Vitamin B2	Ariboflavinosis, insomnia, dermatitis
Vitamin A	Acne, night blindness, insomnia, fatigue, reproductive difficulties
Vitamin B3	Pellagra, muscle weakness, loss of appetite, inflammation
Vitamin B5	Fatigue, headache, nausea
Vitamin B6	Anemia, Convulsions, headache, fatigue, anorexia, hair loss
Vitamin B12	Pernicious anemia, depression
Choline	Impaired digestion of lipids, build-up of fat in liver, CAD disease, hypertension, stunted growth
Folic Acid	Birth defects, premature graying of hair, fatigue, insomnia
PABA (para-aminobenzoic acid)	Gastrointestinal disorders, nervousness, depression, fatigue, patchy skin
Vitamin C	Bleeding gums, scurvy, bronchial infections, poor digestion, bruising, tooth loss
Vitamin D	Rickets, osteomalacia
Vitamin E	Nerve and red blood cell damage, infertility
Vitamin K	Osteoporosis, low prothrombin
Bio-flavonoids (Quercetin)	Asthma
Co-enzyme Q10 (Ubiquinone)	Allergies, dementia, CAD

Boron	Impaired mental function
Calcium	Eczema, hyperlipidemia, hypertension, dental caries, convulsions, muscle cramps, insomnia
Chromium	Glucose intolerance, fatigue, impaired protein metabolism
Copper	Anemia, hyperlipidemia, baldness, diarrhea, general weakness
Germanium	Chronic viral infection, rheumatic arthritis
Iodine	Mental retardation, fatigue
Iron	Digestive problems, anemia, dizziness, fatigue, hair loss, bone fragility
Magnesium	Tachycardia, insomnia, irritability
Phosphorous	Anxiety, bone pain, fatigue, breathing problems
Selenium	Cancer, heart disease
Silicon	Dementia, osteoporosis
Vanadium	CAD, impaired reproductive ability, kidney disease
Zinc	Acne, delayed sexual maturation, fatigue, hair loss, increased susceptibility to infections
Methionine/Laurine	Allergy, autoimmune disease
Arginine	Impaired hepatic lipid metabolism
Carnitine	Obesity, muscle weakness, heart pain, mental confusion
Cysteine/Cystine	Impaired respiratory function, decreased leukocyte function
GABA (Gamma-amino butyric Acid)	Mental stress
Glutamic Acid	Mental retardation, epilepsy, muscular dystrohy
Glutathione	Atherosclerosis
Isoleucine/Leucine	Blood sugar level instability
Serine	Impaired fatty acid metabolism
Tyrosine	Hypothyroidism

A method for treating a nutrient deficiency of a subject from those listed in Table 1 comprises detecting the nutrient deficiency as provided herein and administering the nutrient, or precursor thereto, to the subject. One of skill in the art, in light of the present disclosure, is able to ascertain proper precursor molecule(s) to treat a nutrient deficiency as detected herein.

Nutritional deficiencies, such as, alcoholism and thiamine deficiency, and niacin deficiency and pellagra, are standard and well known and can be used for validation of methods of the present invention.

A generalized lack of nourishment such as protein, energy, or vitamin deficiency, or combinations thereof, may be present in conditions such as anorexia nervosa, kwashiorkor, or marasmus, for example. The methods provided herein are further useful for determining such generalized conditions when a multitude of nutrient deficiencies are detected.

As used herein, the terms "a" and "an" mean "one or more."

All of the methods disclosed and claimed herein can be made and executed without undue experimentation in light of the present disclosure. While the methods of this invention have been described in terms of preferred embodiments, it will be apparent to those of skill in the art that variations may be applied to the methods and in the steps or in the sequence of steps of the method described herein without departing from the concept, spirit and scope of the invention. More specifically, it will be apparent that certain agents which are both chemically and physiologically related may be substituted for the agents described herein while the same or similar results would be achieved. All such similar substitutes and modifications apparent to those skilled in the art are deemed to be within the spirit, scope and concept of the invention as defined by the appended claims.

CLAIMS

1. A method for detecting a deficiency of a nutrient in a subject, comprising:

obtaining lymphocytes from the subject;

obtaining serum from the subject;

incubating the lymphocytes in the presence of the nutrient and a mitogen for a time period to allow at least about a doubling of lymphocyte number to provide a test sample;

incubating the lymphocytes in the presence of optimal proliferating media and a mitogen for the time period to provide an optimal sample;

incubating the lymphocytes in the presence of the serum and a mitogen for the time period to provide a baseline sample;

counting the lymphocytes in the test sample, the optimal sample, and the baseline sample to produce a test count, an optimal count; and a baseline count;

dividing the test count by the optimal count to obtain a test value;

dividing the baseline count by the optimal count to obtain a baseline value; and

comparing the test value with the baseline value;

wherein when the test value is greater than the baseline value, a deficiency of the nutrient is detected in the subject.

2. The method of Claim 1 where the time period is about 48 to about 72 hours.

3. The method of Claim 1 where the counting is carried out using a hematology analyzer.

4. A method for detecting a deficiency of a nutrient in a subject, comprising:

obtaining lymphocytes from the subject;

obtaining serum from the subject;

incubating the lymphocytes in the presence of the nutrient and a mitogen for a time period less than about a doubling time to provide a test sample;

incubating the lymphocytes in the presence of optimal proliferating media and a mitogen for the time period to provide an optimal sample;

incubating the lymphocytes in the presence of the serum and a mitogen for the time period to provide a baseline sample;

determining size of the lymphocytes in the test sample, the optimal sample, and the baseline sample to produce a test size, an optimal size; and a baseline size; and

comparing the test size with the optimal size to obtain a change value;

comparing the baseline size with the optimal size to obtain a baseline value

wherein when the change value is greater than the baseline value, a deficiency of the nutrient is detected in the subject.

5. The method of Claim 4 where the time period is about 12 hours.

6. The method of Claim 4 where determining size is carried out using a hematology analyzer.

7. The method of Claim 1 or 4 wherein the nutrient is selected from the group consisting of a vitamin, a mineral, an amino acid, a carbohydrate, a fat-related molecule, a metabolite, and an anti-oxidant.

8. The method of Claim 1 or 4 wherein the nutrient is an amino acid and the amino acid is arginine, lysine, cysteine, cystine, glycine, leucine, isoleucine, methionine, serine, threonine, tryptophan, tyrosine, valine, or glutamic acid.

9. The method of Claim 1 or 4 wherein the nutrient is a vitamin and the vitamin is biotin, niacin, thiamine, riboflavin, Vitamin B3, Vitamin B5, pyridoxine, folic acid, cyanocobalamin, Vitamin C, Vitamin D, Vitamin E, Vitamin K, or Vitamin A.

10. The method of Claim 1 or 4 wherein the nutrient is a mineral and the mineral is calcium, magnesium, potassium, zinc, iron, copper, chromium, cobalt, manganese,

nickel, germanium, vanadium, selenium, iodine, phosphorous, selenium, fluoride, boron, or silicon.

11. The method of Claim 1 or 4 wherein the nutrient is a fat-related molecule and the fat-related molecule is choline, inositol, cholesterol, a triglyceride, HDL, LDL, lipid, or an essential fatty acid.

12. The method of Claim 1 or 4 wherein the nutrient is a metabolite and the metabolite is pantothenic acid, carnitine, gamma-amino butyric acid, taurine, para-amino benzoic acid, a bioflavonoid, coenzyme Q10, or glutathione.

13. The method of Claim 1 or 4 wherein the nutrient is an anti-oxidant and the anti-oxidant is Vitamin C, selenium, Vitamin E, or urate.

14. The method of Claim 1 or 4 wherein optimal proliferating media is normal serum.

15. The method of Claim 1 or 4 wherein optimal proliferating media is fetal calf serum.

16. A method for improving immune system function in a subject comprising
performing the method of Claim 1 or 4; and
providing the deficient nutrient to the subject in an amount effective to improve immune system function.

17. A method for detecting and treating a nutrient deficiency of a subject suspected of having a nutrient deficiency as listed in Table 1 comprising performing the method of Claim 1 or 4 to detect the nutrient deficiency and administering the deficient nutrient or precursor thereto to the subject to treat the deficiency.

INTERNATIONAL SEARCH REPORT

International application No.
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A. CLASSIFICATION OF SUBJECT MATTER

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US CL :435/4, 29

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/4, 29

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, CHEMICAL ABSTRACTS, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US, 4,499,064 A (SHIVE) 12 FEBRUARY 1985, see column 20.	1-17
Y	TEAGUE, T. K. ET AL. Analysis of Lymphocyte Activation and Proliferation by Video Microscopy and Digital Imaging. Cytometry. 1993 Vol. 14, pages 772-782, see entire document.	4

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
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Date of the actual completion of the international search

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